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Review

Nutritive metal uptake in teleost fish

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Summary

Transition metals are essential for health, forming integral components of proteins involved in all aspects of biological function. However, in excess these metals are potentially toxic, and to maintain metal homeostasis organisms must tightly coordinate metal acquisition and excretion. The diet is the main source for essential metals, but in aquatic organisms an alternative uptake route is available from the water. This review will assess physiological, pharmacological and recent molecular evidence to outline possible uptake pathways in the gills and intestine of teleost fish involved in the acquisition of three of the most abundant transition metals necessary for life; iron, copper, and zinc.

Key words: divalent metal transporter (DMT1), ferroportin, epithelial sodium channel, epithelial calcium channel, Cu-ATPase, ZnT1, rainbow trout, zebrafish.

Introduction

Transition metals (i.e. copper, zinc, iron, cobalt, selenium, manganese) are essential for the health of most organisms, forming integral components of proteins involved in all aspects of biological function. Their ubiquity is governed by their ability to form a wide range of coordination geometries and redox states, which allows these elements to interact with many cellular entities, performing pivotal roles in cellular respiration, oxygen transport, protein stability, free radical scavenging, and the action of many cellular enzymes, as well as for DNA transcription. However, in excess they are toxic, binding to inappropriate biologically sensitive molecules or forming dangerous free radicals. Consequently, there is a fine balance between metal deficiency and surplus and it is vital for organisms to maintain metal homeostasis *via* balancing absorption and excretion.

Fish are unique among the vertebrates, a consequence of having two routes of metal acquisition, from the diet and from the water. This review will focus on the uptake processes present in the gill and intestinal epithelium of teleost fish for the three most abundant nutritive metals: iron, copper and zinc. The majority of the available literature concerns metal uptake processes in freshwater teleosts, but where appropriate examples exist, information on seawater teleosts will be reviewed. Molecular evidence indicates that transporters for these metals identified in yeast, plants or mammals all show high sequence homology in key functional regions (Rolfs and Hediger, 2001), but to date, none of these transporters have been characterised in fish. However, due to the evolutionary conservation of these proteins between yeast, plants and mammals, it is envisaged that fish metal transporters will also belong to the large iron, copper or zinc metal transporter protein families already identified. This review will combine physiological and molecular data to provide an overview of metal uptake mechanisms in teleost fish.

Iron

Iron is an essential nutrient to almost all organisms. Iron positioning in the haem moiety of haemoglobin increases oxygen binding and carrying capacity, enabling oxygen transfer to all tissues in multicellular organisms. One of iron's key cellular functions is to confer redox activity to the cytochromes involved in respiration, due to its ability to exchange electrons in aerobic conditions. A negative consequence of iron's redox flexibility is that it produces oxygen free radicals that are toxic to the cell. Consequently, in excess, iron can be detrimental to health. In addition, excess waterborne iron may be toxic to fish, due to the formation of iron 'flocs' on the gills, resulting in gill clogging and respiratory perturbations (Peuranen et al., 1994; Dalzell and MacFarlane, 1999).

Teleost fish iron homeostasis

The iron content of fish is, in general, considerably lower than that of other vertebrates (Van Dijk et al., 1975), but the precise daily iron requirements for fish are at present unknown.

Aside from the generally lower levels of iron, it is widely assumed that iron metabolism and function in teleost fish is similar to that in other vertebrates (Lall, 1989). Animals lose iron through defecation and epithelial sloughing, and this loss is compensated for by absorption from the diet. In fact, the regulation of iron homeostasis is governed by intestinal absorption, as a regulated excretory mechanism is not known for iron in higher vertebrates (Andrews, 2000).

Branchial versus *intestinal iron uptake*

The role of the gill or gut in iron uptake is dominated by the chemistry of this compound in the natural environment. Iron is one of the most abundant elements on Earth, but in aerobic environments it is predominantly found as ferric (hydro)oxides that are relatively insoluble at neutral pH, and thus, ionic ferric (Fe^{3+}) concentrations are exceedingly low (Stumm and Morgan, 1996). Consequently, unicellular aquatic organisms have evolved specialised transport mechanisms to obtain sufficient iron to meet metabolic demand. This is particularly pertinent in the expanses of the oceans where free iron may be incredibly low (Martin and Fitzwater, 1988). Marine bacteria and blue-green algae have been shown to excrete extracellular chelators of iron, known as siderophores, with exceedingly high affinities for iron ($\log K_{cond}$=19–23; Wilhelm, 1995), forming part of a high-affinity iron-uptake process (see review by Braun and Killmann, 1999). Iron's insolubility and hydrophilic nature in the aquatic environment would suggest that it is relatively unavailable for uptake by fish from the water *via* the gills, and it has been suggested that the diet meets daily iron requirements (Watanabe et al., 1997).

Despite the improbability of aqueous iron acquisition by fish, a number of reports have indicated that fish can obtain iron from the water. Early work by Roeder and Roeder (1966) on swordtail (*Xiphorous helleri*) and platyfish (*X. maculatus*) showed that rearing of newly hatched fry in water of a low iron content [<18 nmol (1 μg) l^{-1} bioavailable iron], at pH 7–8, and a daily ration that contained <0.07 mg iron, resulted in retarded growth rates. However, if the water was supplemented with >25 μmol (3.7 mg) $FeSO_4$ l^{-1}, the fry showed an enhanced growth rate. This response was not observed if the water was spiked with a similar concentration of ferric nitrate, suggesting that the reduced ferrous form is more bioavailable.

There is only one piece of direct evidence for iron uptake across the gill epithelium using radiotracers. Andersen (1997) exposed brown trout *Salmo trutta* larvae (developmental stages of late-eyed eggs, yolk-sac larvae or start-fed fry) to 6.4 or 636 μmol (0.35 or 35 mg) Fe l^{-1}, added as a combination of $^{59}FeCl_3$ and ferric ammonium citrate. Waterborne iron was unavailable to late-eyed eggs and yolk-sac larvae with low bioconcentration factors (tissue-to-water concentration), indicating that the developing embryos receive sufficient iron from their maternal stores, the yolk. The ferroportin transcript (an intestinal basolateral membrane iron transporter identified in zebrafish *Brachydanio rerio*; see *Intestinal iron uptake*, below, for more details) has been located just below the membrane (syntical layer) of the yolk cell (Donovan et al., 2000), suggesting that it is responsible for iron transport from the yolk to the embryo. In the start-fed fry, the gills begin to develop, taking on a prevalent role in cation acquisition from the water (Li et al., 1995). It is the start-fed fry that accumulate ^{59}Fe added to the water. Mortality was seen in the start-fed fry high-iron group, but it is unclear whether this was due to an enhanced iron uptake from the water, or the precipitation of iron resulting in respiratory perturbations (Peurannen et al., 1994; Dalzell and MacFarlane, 1999).

The nutritional value of waterborne iron compared to dietary iron has not been elucidated, but the gills may play a vital role in iron homeostasis at times of developmental need, for example, after yolk-sac absorption and prior to feeding. How fish acquire this iron, despite the constraint of unfavourable water chemistry, has not been determined and requires further investigation.

Intestinal iron uptake

The form in which iron is presented in the feed has a profound effect on bioavailability. For example, Andersen et al. (1997) have shown that haem-bound iron may be more bioavailable than inorganic iron. In mammals, the haem-iron derived from recycled proteolysis of haemoglobin from the bile may be reabsorbed (Conrad et al., 1999). In mammals a considerable amount of iron is still lost *via* the faeces. This deficit is overcome by acquisition of non-haem bound iron from the diet (Andrews, 2000).

Despite very few mechanistic studies of piscine intestinal iron uptake, it may be possible to predict how iron is taken up from the diet. This assumption is based on molecular evidence. cDNAs have been cloned from fish with high sequence similarity with those genes that encode for iron membrane transport proteins in yeast and mammalian systems (see review by Andrews, 2000).

cDNAs whose sequences show high similarity to the ferrous iron transporters termed solute carrier 11a1 (Slc11a1) and solute carrier 11a2 (Slc11a2), formally known as natural resistance associated macrophage protein 1 (NRAMP1) and NRAMP2, have been cloned in a number of fish species, including carp *Cyprinus cyprinus* (Saeij et al., 1999), rainbow trout *Oncorhynchus mykiss* (Dorschner and Phillips, 1999), zebrafish *Brachydanio rerio* (GenBank accession number AF190508) and sea bass *Morone saxatilis* (GenBank accession number AY008746). But, to date, no definitive proof that these sequences encode for an iron transporter has been provided. Slc11a1 is restricted to the cells of the myeloid lineage and is involved in resistance to pathogens (Forbes and Gros, 2001). The role of Slc11a2 in intestinal iron uptake was identified in two separate laboratories using different methods. Gunshin et al. (1997) used expression-cloning techniques in African clawed frog *Xenopus laevis* oocytes to identify intestinal mRNA that conferred iron uptake. Conversely, Fleming et al. (1997) undertook a positional cloning approach to identify genes responsible for microcytic anaemia (*mk*) in mice, a syndrome characterised by defective intestinal iron transport. Functional studies of this gene revealed that the transporter was

a Fe^{2+}/H^+ symporter, operational in the range of pH 5.5–7. This has subsequently been confirmed in a number of studies using the Caco2 cell line, a model cell culture system for mammalian intestinal function (Han and Wessling-Resnick, 2002; Zerounian and Linder, 2002). The Fe^{2+}/H^+ symporter also transports other divalent metals such as Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, as well as the non-essential Cd^{2+} and Pb^{2+} (Gunshin et al., 1997). Due to its metal promiscuity, the transporter is referred to as Divalent Metal Transporter 1 (DMT1), and this terminology will be used throughout the rest of this review.

The rainbow trout DMT1 transcripts are located in most tissues (Dorschner and Phillips, 1999) including the transport epithelia of the gill, intestine and kidney (N. R. B., personal observation). In mammals, the DMT1 transcript is also found in most tissues, but predominantly in the duodenum (Gunshin et al., 1997). This transcript profile corresponds to the anatomical pattern of mammalian intestinal iron uptake (Gunshin et al., 1997). Furthermore, the lumen fluids of the duodenum are slightly below neutral pH, favouring the functioning of a proton symporter. DMT1 transcript is upregulated in iron-deficient mice and is most abundant in the villus-crypt of the duodenal enterocytes, with transcript levels decreasing along the crypt–tip axis (Trinder et al., 1999). The identification of a 3′ UTR iron response element (IRE) associated with the DMT1 gene gives credence to this protein being regulated by cellular iron levels (Gunshin et al., 1997, 2001).

The way in which the intestine maintains iron in a bioavailable ferrous form is uncertain. During digestion of food the acidic environment of the stomach releases ferric iron from the ingested matrix (Powell et al., 1999a; Whitehead et al., 1996). The ferric iron may be bound to mucin that may act to maintain metal solubility in the small intestine (Whitehead et al., 1996). How Fe^{3+} is physically presented to the intestinal tissue is unclear, given that it would first have to traverse the mucus layer covering the epithelium before being taken up. The identification of a mammalian ferric reductase present on the apical membrane of the duodenal enterocytes provides further evidence that iron is imported into these cells *via* a Fe^{2+} transport process (McKie et al., 2001). In addition, ferric iron may also be reduced *via* the presence of reducing agents in the diet, such as ascorbate (Raja et al., 1992). Maintaining an environment that aids ferrous iron uptake *via* a Fe^{2+}/H^+ symporter will be particularly pertinent to marine fish whose intestinal lumen chemistry differs from that of freshwater fish and terrestrial vertebrates (Wilson, 1999).

The intestine of marine fish secretes large quantities of bicarbonate, resulting in the precipitation of divalent cations (Walsh et al., 1991). This secretion may play a role in osmoregulation of marine teleosts (see review by Wilson, 1999). The presence of HCO_3^- at concentrations in excess of 50 mmol l^{-1} (Wilson, 1999) may limit the bioavailability of Fe^{2+}, *via* the precipitation of $Fe(HCO_3)_2$. In addition, a consequence of a large HCO_3^- secretion is an alkaline lumen, which would result in a proton gradient incapable of providing the driving force for Fe^{2+} uptake *via* a proton symporter. Despite such an adverse environment we recently showed that the European flounder *Platichthys flesus* intestine preferentially absorbed ferrous iron when compared to ferric iron (Bury et al., 2001). Flounder intestinal Fe^{2+} uptake occurred predominantly in the posterior region, which differs from the scenario in mammals where uptake is in the anterior region (Trinder et al., 1999). This ferrous iron uptake process was enhanced in fish with low iron status (i.e. low haematocrit), indicating a physiologically regulated process (Bury et al., 2001). It is not known how marine fish maintain Fe^{2+} availability, but it is hypothesised that epithelial mucus secretion may play a role in maintaining metal solubility in fish (Glover and Hogstrand, 2002a), as well as a key role in modulating the microclimate adjacent to the tissue, making this environment suitable for metal transport (Powell et al., 1999a,b).

The passage of iron from the enterocyte into the blood has recently been discerned. It consists of an iron-regulated transporter, which was initially identified by three independent groups, and thus has been termed IREG1 (McKie et al., 2000), MTP1 (Abboud and Haille, 2000) or ferroportin (Donovan et al., 2000). Ferroportin was identified by positional cloning of the gene responsible for hypochromic anaemia in the zebrafish mutant *weissherbst* (Donovan et al., 2000).

The study of Donovan et al. (2000) was originally devised to utilise the concept of ‘model hopping’. Here genetic information from zebrafish was used to identify the genes in humans that are responsible for iron deficiency or overload disorders. The success of this study provides strong evidence that the machinery for cellular iron export is evolutionarily conserved between fish and mammals. Ferroportin is located on the basolateral membrane of the enterocytes (McKie et al., 2000), and export of iron *via* this transporter depends on the presence of a membrane-associated copper containing oxidase, termed haephestin (Vulpe et al., 1999). Iron is transported out of the cell as Fe^{2+}, haephestin oxidises Fe^{2+} to Fe^{3+}, which then binds to transferrin. Transferrin is present in fish (Tange et al., 1997) and in this form the iron is transported to other tissues in the body (McKie et al., 2000). The presence of a 5′-UTR IRE associated with the IREG1/ferroportin gene demonstrates that expression may be regulated *via* cellular iron concentrations (Donovan et al., 2000; McKie et al., 2000).

Branchial iron uptake

The localisation of the DMT1 transcript to the gill epithelium (N. R. B., personal observation) and the evidence for iron being taken up by the gill (see *Branchial* versus *intestinal iron uptake*, above) would suggest that the machinery for iron uptake is present. It is not clear, however, how fish acquire iron with remarkably low concentrations in the water. The gills do not secrete siderphore ‘like’ proteins, but the bacteria (*Vibro* sp.) present on the gills do (Muiño et al., 2001). It is possible that the compounds that make up branchial mucus play a key role in sequestering waterborne iron, but this needs validation. Mucus does act as a barrier on the gill enabling a microclimate close to the tissue to form, and

this may be sufficiently different from the surrounding water to enable apical membrane iron uptake.

A diagrammatic representation of the generic cellular iron uptake pathways in teleost fish, which combines information for both the branchial and intestinal uptake routes described above, is given in Fig. 1.

Copper

Copper acts as a cofactor for a number of key proteins (i.e. superoxide dismutase, ceruloplasmin). As with iron, copper's flexible redox state means it plays a vital role in cellular respiration, with cytochrome *c* oxidase being an important copper protein. Copper is thus an essential element, and daily dietary requirements for fish are in the region of 15–60 μmol (1–4 mg) Cu kg^{-1} dry mass (Lanno et al., 1985; Watanabe et al., 1997). However, in excess, copper is toxic. From a dietary perspective the primary toxic action is predominantly the production of free radicals in tissues where copper accumulates. In addition, dietary copper toxicity can occur at several other loci in the gut and includes inhibition of digestive enzymes and reduced gut motility (Woodward et al., 1995). Conversely, high concentrations of waterborne copper affect branchial function, the main toxic action being a perturbation of sodium homeostasis (Laurén and McDonald, 1985). For recent reviews on the toxicity of dietary and waterborne copper, see Clearwater et al. (2002) and Wood (2001), respectively.

Teleost fish copper homeostasis

Plasma copper levels are tightly regulated in the freshwater rainbow trout (Grosell et al., 1997). As with mammals (Harris, 2000), the liver is the major organ involved in copper homeostasis (Grosell et al., 1997, 2000; Kamunde et al., 2001, 2002a). The liver accumulates a large proportion of the copper absorbed from the diet or water, and is the site for synthesis of the most abundant copper-containing protein in the body, ceruloplasmin. Ceruloplasmin is secreted into the blood and acts as a source of copper to extra-hepatic organs (Harris, 2000). Copper may also circulate in the body bound to albumin and other low-molecular mass proteins (Harris, 2000). The main site for secretion of excess copper in teleost fish is *via* the bile (Grosell et al., 1997, 2000) and, in the case of the European eel *Anguilla anguilla*, very little copper is found in the urine (Grosell et al., 1998). The gills of fish have also been implicated in copper excretion (Handy, 1996), but this has yet to be fully characterised. In fish, the mechanism by which excess copper is transported across the cannicular membrane of the liver into the bilary ducts has not been ascertained. In mammals there are three candidate secretory pathways: (1) a Cu-ATPase, identified in patients suffering from Wilson's disease, which is a hereditary disorder that results in elevated plasma copper concentration, due to the inability of the body to secrete copper *via* the Cu-ATPase (termed Wilson's protein or ATP7B) (Bull et al., 1993; Harris, 2000; Puig and Thiele, 2002); (2) a multiorganic cation transporter (cMoat) (Elferink and Jansen, 1994) and (3) lysosomal secretion (Gross et al., 1989).

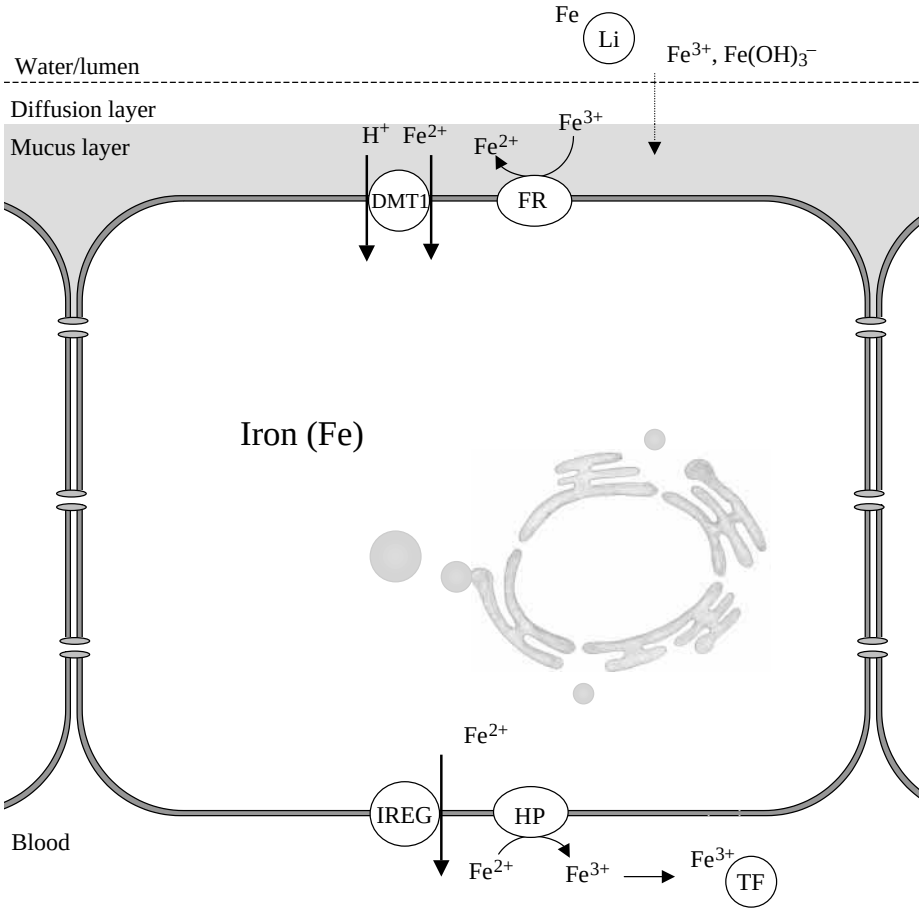

Fig. 1. Hypothetical representation of cellular iron uptake pathways in fish combining data from gill and intestine. See text for more details. Briefly, ferric iron (Fe^{3+}) is reduced *via* an apical membrane bound ferric reductase (FR). Ferrous iron (Fe^{2+}) enters the cell *via* a Fe^{2+}/H^{+} symporter (DMT1). Basolateral Fe^{2+} export occurs *via* an iron regulated transporter (IREG1), also known as ferroportin. IREG1 is linked to a membrane-bound copper-containing oxidase, termed hephaestin (HP), that oxidizes Fe^{2+} to Fe^{3+}. Fe^{3+} binds to transferrin (TF) in the blood. Li, aquatic ligand.

Branchial versus *intestinal copper uptake*

The diet is the major source of copper for fish under optimal growth conditions (Handy, 1996; Kamunde et

al., 2002a,b). It is evident from waterborne toxicity studies that the gill can also contribute considerably to copper uptake (Taylor et al., 2000). Studies by Miller et al. (1993) and Kamunde et al. (2002a) have highlighted the significance of waterborne copper as a potential nutritional source to rainbow trout. In the latter study, rainbow trout fry were fed either a low [12.5 nmol (0.8 μg) Cu g^{-1}], normal [50 nmol (3.2 μg) Cu g^{-1}], or high [4390 nmol (281 μg) Cu g^{-1}] copper diet, in combination with either low [6.25 nmol (0.4 μg) Cu l^{-1}] or normal [47 nmol (3 μg) Cu g^{-1}] waterborne copper regimes. By utilising the copper radionuclide, ^{64}Cu, the investigators were able to ascertain the relative significance of dietary or waterborne routes for newly accumulated copper in these various groups. The fish fed with a low-copper diet and kept in low-copper water showed a marked reduction in growth over a 50-day experimental period. The growth rate of those fish fed the same diet but reared in normal waterborne copper levels showed no changes compared to the other groups, and 60% of the copper accumulated by these fish was from waterborne copper. In contrast, the dietary source of copper accounted for 99% of the accumulated copper in those fish fed on a high-copper diet. Miller et al. (1993) concluded that the diet was also the major source of copper for rainbow trout, the copper accumulated from the waterborne route accounting for 37% of the liver copper burden. These studies demonstrate the significance of waterborne copper for fish health at times when the dietary source of copper may be inadequate.

Intestinal copper uptake

In fish there is evidence that apical entry of copper into the intestinal epithelium is a passive process, and the rate-limiting step of intestinal copper uptake is basolateral membrane extrusion (Clearwater et al., 2000; Handy et al., 2000). This conclusion is supported by two independent observations. Clearwater et al. (2000) noted a Q_{10} ratio of <1 for copper accumulation into the epithelium of rainbow trout intestine, and Handy et al. (2000) observed a dose-dependent accumulation of copper into the intestinal mucosa of the African walking catfish *Clarias gariepinus*, but no such relationship between lumen copper concentrations and the appearance of copper into the blood. Passive diffusion may also occur in mammals (Crampton et al., 1965). Other uptake pathways may be present, such as the copper entry *via* an amiloride-sensitive Na^+ pathway observed in rat intestine (Wapnir, 1991). Caution is required, however, when interpreting metal uptake studies in the presence of amiloride, because this drug may form metal-complexes that are unavailable to the organism (*viz* Bury and Wood, 1999; Grosell and Wood, 2002).

It is of interest that mammalian intestinal copper uptake primarily occurs in the small intestine (Wapnir and Stiel, 1987), whereas in fish, copper uptake is found on the mid/posterior region (Clearwater et al., 2000; Handy et al., 2000). The same disparity between the positioning of the iron uptake pathways in fish and mammals has also been observed (Bury et al., 2001).

At present there are two proposed mechanisms of basolateral Cu transport in fish: (1) a Cu P-type ATPase and (2) a Cu/anion symporter (Handy et al., 2000). The Cu-ATPase involved in mammalian intestinal copper uptake was identified from patients with Menkes (MNK) syndrome. This genetic condition results in low plasma copper levels due to a defect in the MNK protein (termed ATP7A) involved in copper transport from the enterocytes to the blood (Vulpe et al., 1993). The MNK cDNA shows similarities to a number of other Cu-ATPases in bacteria (Solioz and Odermatt, 1995; Mandal et al., 2002), yeast (Riggle and Kumamoto, 2000) and mammals (Vulpe et al., 1993; Qian et al., 1998). The evolutionarily conserved nature of this protein would suggest its presence in fish, and support for this comes from the recent identification of a partial cDNA homologue to the MNK protein in the Gulf toadfish *Opsanus beta* (Grosell et al., 2001).

Under normal conditions, copper that enters cells from the lumen is bound to intracellular metallochaperones, resulting in intracellular 'free' copper levels as low as 10^{-18} mol l^{-1} (1 attomole; Huffman and O'Halloran, 2000). Metallochaperones traffic the metal to sites within the cell where it is incorporated into cuproproteins (see reviews by O'Halloran and Culotta, 2000; Puig and Thiele, 2002). An example is the human metallochaperone, HAH1 (Klomp et al., 1997), which delivers monovalent Cu [Cu(I)] to the Golgi apparatus, where it donates Cu(I) to the MNK protein (Huffman and O'Halloran, 2000). Cu(I) is transported *via* this Cu-ATPase into the lumen of the Golgi. Vesicles containing Cu(I) bud off the Golgi network and are redistributed to the basolateral membrane where Cu(I) is secreted from the cell (Petris et al., 1996; Francis et al., 1999). The MNK protein is then recycled (Petris and Mercer, 1999). A similar trafficking process is probably present in fish.

In the presence of excessive (possibly toxic) dietary levels, copper is prevented from entering the body by retention in the gut tissue bound to the small molecular mass cysteine-rich proteins, i.e. metallothionein (MT) (Olsen et al., 1996). Potentially, this MT-bound copper may then be excreted into the faeces *via* sloughing of the epithelial membrane (Handy, 1996; Clearwater et al., 2000).

Evidence for a Cu/anion symporter extrusion process in fish intestine comes from experiments performed on isolated everted gut sacs from the African walking catfish (Handy et al., 2000). In these studies, applications of drugs designed to inhibit P-type ATPases, vanadate (Cantely et al., 1978), and a Cl^-/HCO_3^- antiporter, DIDS, stimulated Cu transport from the tissue to the serosal medium. At first the lack of inhibition by vanadate appears puzzling, because the MNK protein is a P-type ATPase. However, very little vanadate may have been in contact with functional Cu-ATPases at the Golgi membrane because of the slow movement of vanadate across the intestine where the muscle layer is still intact (Handy et al., 2000), and intracellular bioreactive vanadate concentrations may be reduced due to chelation (Edel and Sabbioni, 1993). The stimulation of copper efflux by this drug was proposed to be due to a reduction in the transepithelial potential in the

presence of vanadate. This resulted in a reduction in the electrochemical gradient leading to enhanced copper movement (Handy et al., 2000). The stimulation by DIDS was proposed to be a consequence of the rise in intracellular [Cl^-] resulting from inhibition of the basolateral membrane Cl^-/HCO_3^- antiporter. This observation, along with the fact that copper efflux reduction is coupled to a decrease in mucosal [Cl^-], suggests the presence of a basolateral Cu/Cl^- symporter. Metal ion/Cl^- symporters have been observed in other cell types (Torrubia and Garay, 1989; Alda and Garay, 1990; Endo et al., 1998; Ödblom and Handy, 1999). This may be a novel mechanism by which copper traverses vertebrate intestine, and further research is required to determine the precise mechanism(s) of teleost fish intestinal basolateral membrane Cu extrusion.

Branchial copper uptake

A recent paper by Grosell and Wood (2002) has identified two branchial apical copper uptake processes, a sodium-sensitive and a sodium-insensitive pathway. Both uptake pathways showed saturation kinetics with similar low affinities for Cu (K_m 7.1 nmol l^{-1} for the sodium-sensitive and 9.5 nmol l^{-1} Cu for the sodium-insensitive pathways). The sodium-sensitive copper uptake pathway was characterised by an IC_{50} of 104 µmol l^{-1} sodium, but copper uptake was not completely inhibited in the presence of 20 mmol l^{-1} sodium. In addition, the sodium-sensitive pathway was inhibited by the drugs phenamil (an amiloride analogue that is an irreversible inhibitor with high affinity to epithelial sodium channels, ENaCs) (Kleymann and Cragoe, 1988) and bafilomycin A (a proton pump inhibitor) (Drose and Altendorf, 1997). This suggests that copper is entering *via* a putative ENaC. Coincidently, the non-essential metal monovalent Ag [Ag(I)], which has been shown to mimic Cu(I) in various transport processes (Solioz and Odermatt, 1995; Havelaar et al., 1999; Riggle and Kumamoto, 2000; Mandal et al., 2002) has also been shown to enter fish *via* a sodium uptake pathway (Bury and Wood, 1999). However, the biophysical characteristics of known ENaCs show that they allow only the passage of Na^+ and the smaller Li^+ (Garty and Palmer, 1997). Consequently, the proposition that apical copper or Ag(I) entry is *via* a branchial Na^+ channel (Bury and Wood, 1999), suggests that the teleost ENaC possesses unique characteristics.

The nature of the branchial sodium-insensitive copper uptake pathway is unclear, but the identification of high-affinity copper importers (the Ctr family of proteins) in evolutionarily distinct organisms, such as yeast (Dancis et al., 1994) and mammals (Zhou and Gitschier, 1997; Lee et al., 2002) may provide clues. The human Ctr1 (hCtr1) has, however, a much lower affinity (K_m 1.71–2.54 µmol l^{-1} Cu, based on vector–cell transfection studies; Lee et al., 2002), than the uptake of Cu across the fish gill (9.5 nmol l^{-1} Cu; Grosell and Wood, 2002). The disparity may be simply because the assays performed on fish were carried out in ion-poor water where the copper is present almost exclusively (90%) in the ionic form, whereas in cell culture conditions copper will be bound to various components of the culture medium (cf. Grosell and Wood, 2002). Re-examination of apical Ag(I) uptake in rainbow trout suggests that there is also a proportion of Ag(I) uptake that is sodium-insensitive (Bury and Wood, 1999). A 260,000-fold excess of water Na^+ could not exclusively prevent branchial Ag(I) uptake. This may suggest that Ag(I) and Cu(I) share both the sodium-sensitive and sodium-insensitive uptake pathways. However, a 100-fold excess of copper is required to prevent Ag(I) from entering rainbow trout, which suggests that if this uptake pathway is shared, it has a higher affinity for Ag(I) compared to copper (Bury and Hogstrand, 2002).

Interestingly, the close relationship between copper and

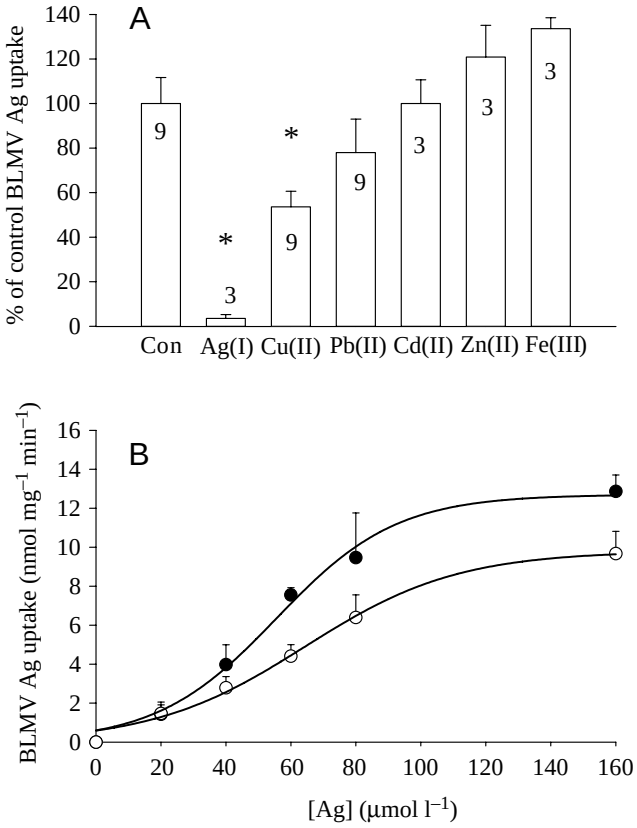

Fig. 2. (A) Inhibition of rainbow trout gill basolateral membrane vesicle (BLMV) Ag(I) transport at 50 µmol l^{-1} $AgNO_3$ by 500 µmol l^{-1} of non-radioactive Ag(I), Cu(II), Pb(II), Cd(II), Zn(II) and Fe(III). Values are means ± S.E.M., numbers indicate *N* values. Asterisks indicate significant differences from control values (Student *t*-test performed on arcsine transformed data, $P<0.05$). (B) Concentration-dependent Ag(I) transport (solid circles), and in the presence of 800 µmol l^{-1} Cu(II) (open circles). Values are means ± S.E.M., $N=6$, taken from two separate experiments. Both sets of data best-fitted a sigmoidal curve with regression equations: for the control vesicle Ag(I) transport, $y=3.5\pm2.1/(1+e^{-(x-15.1\pm3/17.5\pm2.4)})$, $r^2=0.992$; for Ag(I) transport in Cu(II)-treated vesicles, $y=9.8\pm1.6/(1+e^{-(x-17.4\pm2.9/6.4\pm3.5)})$, $r^2=0.993$. The Cu(II)-treated vesicle Ag(I) transport is significantly reduced (two-way analysis of variance, $P=0.0052$). The protocol for BLMV preparation and transport buffer media were taken from Bury et al. (1999).

silver uptake is also seen with hCtr1, where copper uptake is significantly blocked by Ag(I) (Lee et al., 2002). This implies that copper is entering *via* the hCtr1 in the monovalent form. Copper is predominantly found as the Cu(II) valency in water. Thus, to enter *via* a putative Na^+-channel or Ctr carrier, Cu(II) must be reduced to Cu(I). The presence of a copper-reductase on the gills of fish however, has not been shown.

Branchial basolateral copper extrusion occurs *via* a carrier mediated process (Grosell et al., 1997; Campbell et al., 1999). Using an *in situ* perfused head technique, Campbell and coworkers demonstrated second-order reaction kinetics for the movement of copper from the gills of rainbow trout into the perfusate. This copper transport was inhibited by vanadate, suggesting the involvement of a P-type ATPase. The concern over whether vanadate is bioreactive within the cell (see *Intestinal copper uptake*, above, for details) means it is unclear whether this active branchial copper ATPase is resident at the Golgi (i.e. akin to the MNK protein) or at the basolateral membrane. However, a Ag(I)stimulated ATPase has been identified in basolateral membrane vesicles (BLMV) prepared from the gills of rainbow trout (Bury et al., 1999). Inhibition studies of BLMV Ag(I) uptake by various metals (Cu, Pb, Cd, Zn, Fe) show that copper is the only antagonist (Fig. 2A). This is further verified by the inhibition of dose-dependent BLMV Ag(I) uptake by copper (Fig. 2B). The inference from these studies is that the fish gill basolateral membrane Ag(I) transporter (Bury et al., 1999) is in fact a Cu(I) P-type ATPase, and Ag(I) may simply be mimicking Cu(I). Considering that there is only partial contamination of fish gill BLMV with the Golgi membrane marker thiamine pyrophosphatase (Perry and Flik, 1988) this would argue against the possibility of Ag(I) mimicking Cu(I) for transport *via* a MNK ATPase residing in

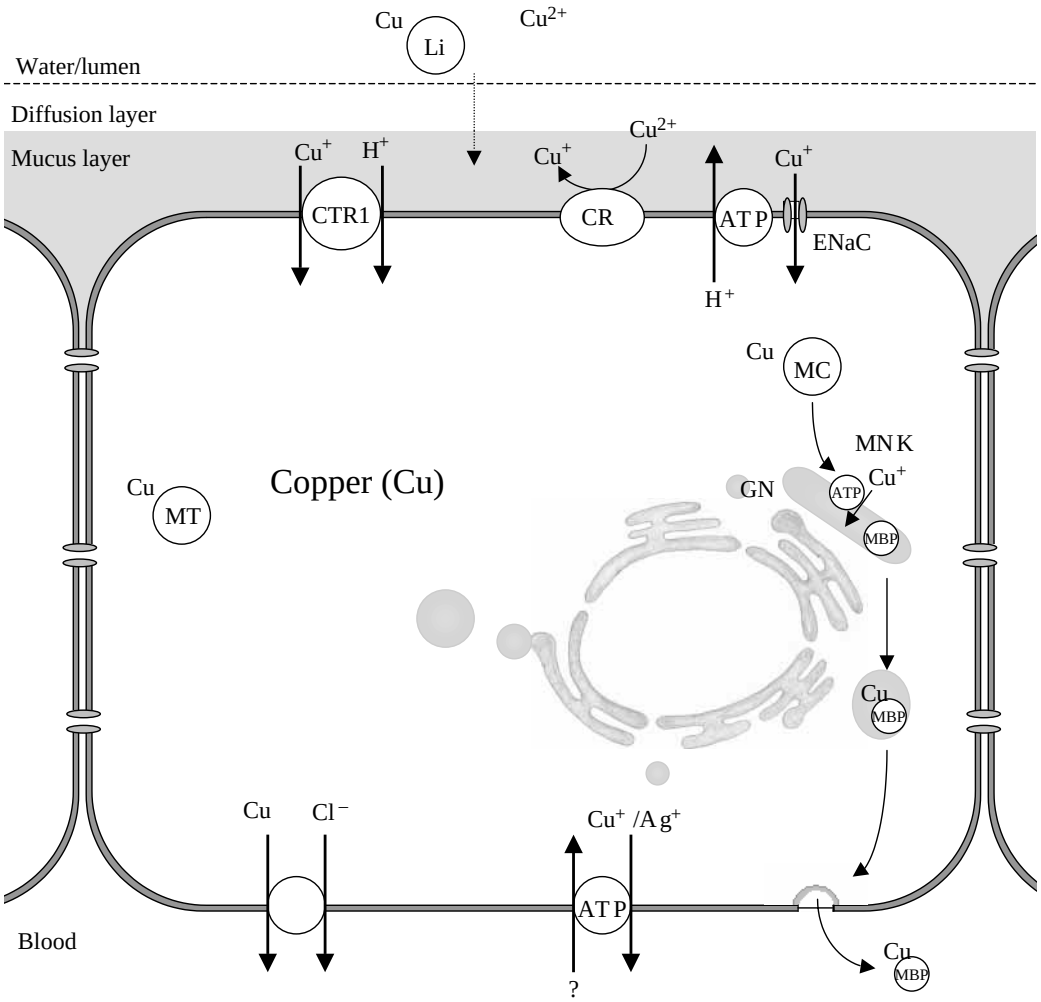

Fig. 3. Hypothetical representation of cellular copper uptake pathways in fish, combining data from gill and intestine. See text for more details. Briefly, in the gills, cupric copper is probably reduced to Cu^+ and enters *via* either a putative epithelial sodium channel (EnaC) or copper transporter 1 (CTR1). Metallochaperones (MC) bind Cu^+ and guide it to the Golgi network (GN), where it is transported into the Golgi lumen *via* a MNK-'like' Cu^+-ATPase. Cu^+ is incorporated into metal binding proteins (MBP) within the GN. GN vesicles then traffic the copper to the basolateral membrane for release *via* exocytosis. Other ATPases on the basolateral membrane exporting copper (i.e. Ag^+/Cu^+-ATPase) may also be present. In the intestine, apical entry is presumed to be passive. Intestinal export may be *via* a Cu/Cl^- symporter, or *via* the MNK pathway described above. Excess copper is bound to low molecular mass proteins, such as metallothionein (MT). Li, aquatic ligand; MNK, Menkes Cu^+-ATPase; CR, copper reductase.

the trans-Golgi network, and it may be possible that a teleost MNK-'like' protein is functional at the basolateral membrane.

Fig. 3 combines information for both the branchial and intestinal uptake routes described above, and represents the generic epithelial copper uptake pathways in teleost fish.

Zinc

Zinc is essential due to its vital structural and/or catalytic importance in more than 300 proteins that play important roles in piscine growth, reproduction, development, vision and immune function (Watanabe et al., 1997). Consequently for fish, of the essential metals, zinc is second in quantitative importance only to iron (Watanabe et al., 1997). Dietary zinc requirements range between 230–460 μmol (15–30 mg) kg^{-1} dry mass of diet (Ogino and Yang, 1978; Gatlin and Wilson, 1983).

The ubiquity of zinc is governed by its ability to form a wide range of coordination geometries, allowing it to interact with a wide range of cellular entities (Vallee and Falchuk, 1993; McCall et al., 2000). Furthermore zinc is redox inert, enabling the formation of relatively stable associations within the cellular environment (Vallee and Falchuk, 1993). Consequently, in contrast to copper and iron, zinc does not form free radical ions, and in fact has antioxidant properties (Powell, 2000). Zinc may, however, generate toxicity to fish by interfering with calcium homeostasis (Spry and Wood, 1985; Hogstrand and Wood, 1996).

Teleost fish zinc homeostasis

At both organismal and cellular levels zinc status is tightly controlled. Surplus zinc is either excreted *via* the bile, intestinal sloughing (Handy, 1996) or the gills (Hardy et al., 1987), whilst urinary loss of zinc in fish is minimal (Spry and Wood, 1985). Even though it has been proposed that excretion is the main means by which fish control body zinc homeostasis (Shears and Fletcher, 1983; Hardy et al., 1987), they are also able to regulate zinc acquisition. The proportion of zinc absorbed from the diet decreases as the dietary zinc load increases (Shears and Fletcher, 1983; Hardy et al., 1987; Glover and Hogstrand, 2002b), suggesting the presence of a mechanism for regulating uptake of dietary zinc. Branchial zinc accumulation is also regulated, and rainbow trout exposed to elevated waterborne zinc levels show alterations in zinc uptake mechanisms that limit the amount of zinc accumulating on the gill (see Branchial zinc uptake; Hogstrand et al., 1994, 1995, 1996, 1998). In a similar way, mammals adjust zinc absorption and endogenous intestinal zinc excretion to maintain zinc status (King et al., 2000).

Branchial versus *intestinal zinc uptake*

The major routes of zinc assimilation in fish are the gills and the gut. The relative importance of these routes has been the focus of much research in both marine (e.g. Pentreath, 1973; Renfro et al., 1975; Milner, 1982; Willis and Sunda, 1984) and freshwater fish (Spry et al., 1988). The consensus is that the gut is the dominant pathway of absorption in the natural environment. With decreasing dietary zinc levels, however, the gill may become increasingly important, especially when waterborne zinc levels are elevated (Spry et al., 1988). Hence the intestine appears to act as the bulk pathway for uptake, whereas the gills may act to supplement absorption when required.

The relative zinc uptake affinities and capacities of gill and gut appear to confirm this scenario in freshwater rainbow trout. The affinity (K_m) for branchial zinc uptake lies between 3.6 and 7.9 μmol l^{-1} (Spry and Wood, 1989; Hogstrand et al., 1998). The corresponding constant for the intestine is 309 μmol l^{-1} (Glover and Hogstrand, 2002b), indicating a lesser affinity for zinc. However the gut appears to have a much greater capacity for zinc uptake with a maximal rate (J_{max}) of 933 nmol kg^{-1} h^{-1} (Glover and Hogstrand, 2002b), compared to 240–410 nmol kg^{-1} h^{-1} for the gill (Spry and Wood, 1989; Hogstrand et al., 1998). Interestingly, the unicellular organism yeast has been demonstrated to have independently regulated high- and low-affinity zinc transporters (Zhao and Eide, 1996a,b), a cellular equivalent of the organ-level patterns observed in fish.

Intestinal zinc uptake

In general, the site of gastrointestinal zinc absorption appears conserved between fish and mammals. Pentreath (1976) and Shears and Fletcher (1983) determined that the anterior intestine was the most important region for zinc absorption in winter flounder *Pseudopleuronectes americanus* and plaice *Pleuronectes platessa*, respectively. This is consistent with the scenario in human intestine, which exhibits a jejunal-biased absorptive pattern (Lee et al., 1989).

Shears and Fletcher (1983) described two components of uptake in winter flounder. One saturable component dominated at low zinc levels, with a diffusive pathway more dominant at higher zinc concentrations. This mimics the mechanism of zinc uptake in mammals (Lönnerdal, 1989). However, in freshwater rainbow trout, only a saturable component of uptake was discerned using an *in vivo* perfusion technique (Glover and Hogstrand, 2002b). It was proposed that any potential diffusive uptake pathway was blocked as a consequence of increased epithelial mucus secretion in intestine perfused with high zinc concentrations (Glover and Hogstrand, 2002b). In contrast, at low zinc levels, mucus may in fact enhance zinc uptake by trapping zinc close to the epithelial surface, and potentially increasing bioavailability (Powell et al., 1999a). But, at environmentally relevant intestinal zinc concentrations (i.e. up to approx. 50 μmol l^{-1}; Turner and Olsen, 2000; Farag et al., 2000), any diffusive component of zinc uptake is unlikely to be of importance for nutritive zinc uptake.

The apical entry steps in fish intestinal zinc absorption have not been elucidated. In recent years the molecular characterisation of zinc metal importers from evolutionary diverse organisms (yeast, plants and mammals) has been achieved (Zhao and Eide, 1996a,b; Grotz et al., 1998; Gaither and Eide, 2001a), and these proteins form the large ZIP family

of transporters (derived from Zrt, Irt-like proteins; Lioumi et al., 1999). It is highly likely that teleosts possess ZIP homologues. An alternative candidate for intestinal apical zinc absorption has recently been identified by Cragg et al. (2002) termed hZTL, and is related to the zinc transporter (ZnT-1) involved in zinc export described below.

A number of small molecular mass ligands in the enterocyte cytoplasm may modulate piscine zinc uptake. A role for metallothionein (MT) in cellular zinc uptake and metabolism has been proposed (Shears and Fletcher, 1979, 1983, 1984), and evidence from mammalian systems suggests that MT functions in nutritive uptake in zinc-deficient animals (Hoadley et al., 1988; Coyle et al., 2000). In addition, glutathione also has an important role in zinc uptake (Jiang et al., 1998), and the presence of zinc bound to low molecular mass ligands following dietary zinc exposure has been noted in freshwater rainbow trout (Spry et al., 1988). The sequestering molecules such as MT and glutathione act to maintain intracellular 'free' zinc (Zn^{2+}) concentrations in the femtomolar range (Outten and O'Halloran, 2001).

Recently, Glover et al. (2002) have showed that intestinal basolateral transfer of zinc is *via* a saturable pathway in the Gulf toadfish. This contrasts to the finding of Shears and Fletcher (1983) that demonstrated a passive movement of zinc across the winter flounder intestine. A facilitated zinc export process in fish is supported by the cloning of a full-length cDNA in the puffer fish *Fugu ribrepes* with amino acid sequence similarities to the Zinc transporter-1 (ZnT-1) protein of mammals (Balesaria and Hogstrand, 2001). ZnT-1 is localised to the basolateral membrane of enterocytes and is involved in the export of zinc from the intestine into the blood stream (Cousins and McMahon, 2000). Whether the piscine ZnT1 is involved in the regulation of zinc uptake awaits verification.

Aquaculture studies have tended to focus more on endpoints of zinc uptake (i.e. growth) rather than on the mechanism of uptake. These studies, however, have provided interesting information from a mechanistic perspective. In particular, the chemical form of zinc added to diets has been the focus of a number of investigations. Some authors describe enhanced body zinc status with amino acid chelates (Hardy et al., 1987; Paripatananont and Lovell, 1995; Apines et al., 2001), whereas others report no effect (Li and Robinson, 1996). Amino acids with high affinity for zinc enhance bioavailability, and physiological studies have shown that histidine and cysteine may increase zinc acquisition, probably *via* specific uptake pathways related to the formation of bis complexes with zinc [$Zn(His)_2$, $Zn(Cys)_2^{2-}$; Glover and Hogstrand, 2002a]. In addition, the chelation of zinc by amino acids may, by altering the distribution of internal zinc, have nutritional benefits (Glover and Hogstrand, 2002a).

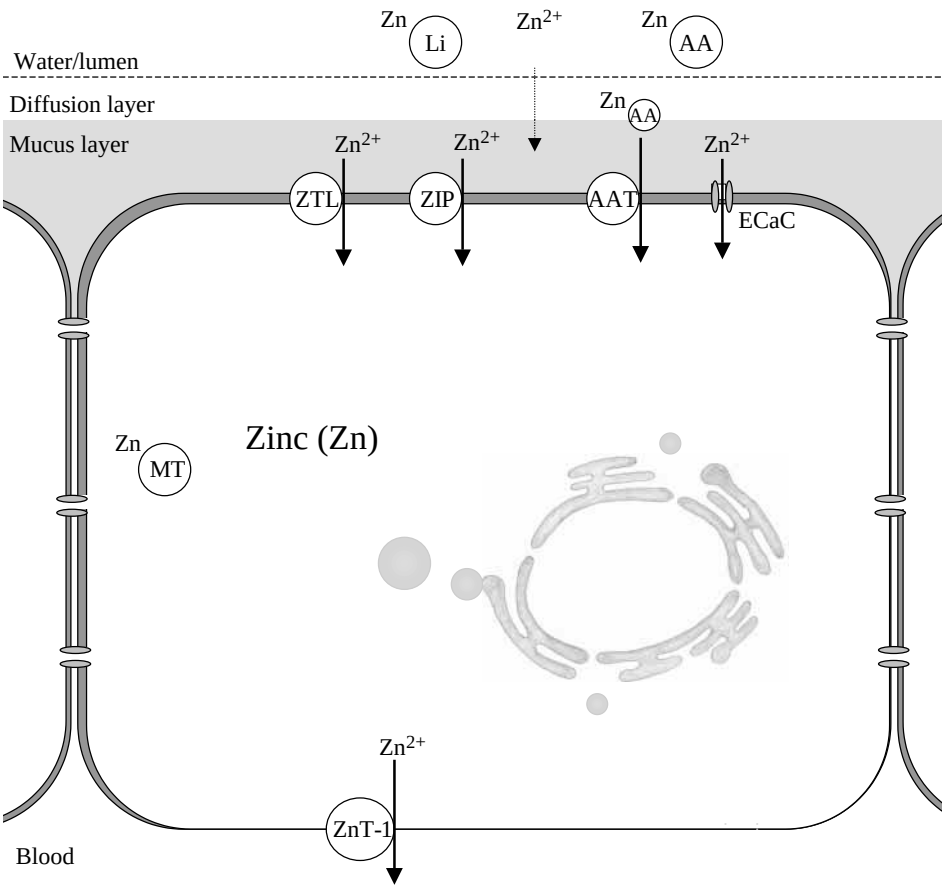

Fig. 4. Hypothetical representation of cellular zinc uptake pathways in fish combining data from gill and intestine. See text for more details. Li, aquatic ligand; ZIP1, Zrt, Irt-like protein; EcaC, putative lanthanum-sensitive epithelial calcium channel; ZTL, zinc-regulated zinc transporter; AA, amino acid; AAT, amino acid transporter; ZNT1, zinc transporter 1. Briefly, zinc enters *via* either a putative calcium channel, a ZIP-'like' transporter, ZTL a zinc transporter similar to zinc transporter 1 (ZnT-1), or bound to an amino acid (i.e. histidine), *via* an amino acid transporter. Excess cytoplasmic zinc is bound to metallothionein (MT). Basolateral transfer is *via* a Znt-1.

Branchial zinc uptake

The mechanism of freshwater branchial zinc uptake is now well understood. Many investigations have shown that hardness (i.e. water [Ca^{2+}]) offers a protective effect against waterborne zinc toxicity (e.g. Eisler, 1993). The relationship between calcium and zinc homeostasis is also apparent at the branchial apical uptake step. Numerous studies have shown that calcium inhibits branchial zinc uptake (Spry and Wood, 1989; Bentley, 1992; Hogstrand et al.,

1996), and correspondingly, that zinc competes with calcium uptake. Injection of stanniocalcin, a hypocalcaemic hormone in fish (Wagner et al., 1986), downregulates both calcium (Flik et al., 1993) and zinc uptake from the water in rainbow trout (Hogstrand et al., 1996). In addition, lanthanum, a calcium channel blocker, also inhibits both calcium (Perry and Flik, 1988) and zinc uptake (Hogstrand et al., 1996). Calcium has also been observed to compete for zinc uptake *via* a channel present in the brush border membrane of the pig intestine (Bertolo et al., 2001). It would thus appear that zinc uptake occurs *via* a lanthanum-sensitive Ca^{2+}-channel, which has been located in the branchial chloride cells (Perry and Flik, 1988). It is of interest that another essential metal, cobalt, has also been shown to enter carp *Cyprinus carpio* gills *via* a Ca^{2+}-channel (Comhaire et al., 1994), suggesting that this channel may discriminate between various divalent cations. It is likely, however, that alternative zinc uptake pathways across the apical surface exist, and interestingly, the affinity constant of *in vitro* zinc transport by ZIPs (see Intestinal zinc uptake) (apparent K_m=3–3.5 μmol l^{-1}; Gaither and Eide, 2001b) corresponds closely to that determined for teleost freshwater fish gill uptake (3.7 μmol l^{-1}; Hogstrand et al., 1995).

The generic cellular zinc uptake pathways in epithelial cells of teleost fish is given in Fig. 4.

Conclusions

The mechanism of branchial metal uptake in marine fish has received little attention. The tendency for decreased metal bioavailability to the gill in marine environments (Rainbow, 1995), and the fact that marine fish could imbibe waterborne metal to compensate for any dietary deficiency, suggests that gastrointestinal uptake is likely to dominate nutritive metal uptake in seawater. Nevertheless, given the vastly different branchial physiology in marine fish, examination of branchial metal uptake would be of considerable interest. Investigations in the marine bivalve *Mytilus edulis* suggest that calcium has a significant influence on branchial zinc uptake (Vercauteren and Blust, 1999). Copper has been shown to influence branchial enzymes in the seawater-adapted European flounder following waterborne exposure (Stagg and Shuttleworth, 1982), but is unclear whether the source of this branchial copper is from the water or diet. Cu/Ca ratios in the otoliths of juvenile barramundi *Lates calcarifer* correlate with the water ratios and not the dietary ratios of these divalent cations (Milton and Chenery, 2001), suggesting that Cu laid down in the otolith originates from the sea. Cloning of a putative Cu-ATPase in the marine Gulf toadfish suggests that the molecular machinery for marine metal uptake across the gills is present (Grosell et al., 2001). However, future research is thus required to determine whether nutritive metals are taken up from the sea *via* the gills of teleost fish, the transport proteins involved in this acquisition process, and whether this route of uptake contributes significantly to metal homeostasis.

The presence of teleost fish homologues of metal transporters (DMT1, ferroportin, Cu-ATPase and ZnT-1) suggests an evolutionarily conserved mechanism of nutrient metal uptake. The development and utilisation of molecular techniques that are currently being applied in mammalian systems should facilitate functional characterisation of the uptake process in fish. In addition, novel piscine uptake processes (i.e. $Cu^{+/2+}/Cl^-$ symporter; a putative Cu/Na epithelial sodium channel) may provide insights into alternative transport mechanisms of these metals in other vertebrates.

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